View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by SEDICI - Repositorio de la UNLP

BIBLIOTECA
JORGE D. WILLIAMS

Reprinted from ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, Volume 108, No. 2, November, 1964
Copyright © 1964 by Academic Press Inc.
Printed in U.S.A.

ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS **108**, 341–348 (1964)

COLECCION HERPETOL...
Y BIBLIOTECA
Dr. José Miguel Cei

Isolation and Amino Acid Sequence of Physalaemin, the Main Active Polypeptide of the Skin of *Physalaemus fuscumaculatus*[1]

A. ANASTASI, V. ERSPAMER, AND J. M. CEI

From the Institute of Pharmacology, University of Parma, Parma; the Farmitalia S.p.A. Research Laboratories, Milan, Italy; and the Institute of Biology, National University of Cuyo, Mendoza, Argentina

Received July 1, 1964

Crude methanol extracts of fresh and dried skins of *Physalaemus fuscumaculatus*, a South American amphibian, were subjected successively to chromatography on alkaline alumina and to countercurrent distribution. The main active constituent of these extracts, the polypeptide *physalaemin*, was thus obtained in a pure form. Total and partial acid hydrolysis, fission with trypsin and chymotrypsin, and end-group determination experiments demonstrated that physalaemin is an endecapeptide having the following amino acid composition and sequence:

Pyr-Ala-Asp(OH)-Pro-Asp(NH_2)-Lys-Phe-Tyr-Gly-Leu-Met-NH_2

Some anomalies in the trypsin and chymotrypsin fission of physalaemin are discussed, and the strict chemical resemblance between physalaemin and eledoisin is pointed out.

INTRODUCTION

Methanol extracts of dry or wet skin of *Physalaemus fuscumaculatus*, a South American amphibian, possess a powerful hypotensive action in some experimental animals, and display a potent stimulant action on several extravascular smooth muscles (1).

The present communication describes the experiments which have led to the isolation of the active principle and to its identification with an endecapeptide having a structure similar to that of eledoisin (2, 3). A preliminary report on this topic has already appeared (4).

MATERIALS AND METHODS

The *Physalaemus* material considered in this study was as follows:

(a) 323 adult specimens captured near Tucuman (Argentina) in January–February, 1963. The dried skins weighed all together 32.8 gm, and the average weight of a skin was 0.10 gm. The physalaemin content was 650 μg per gram dry skin.

(b) 471 adult specimens captured at the same time as the preceding ones, at the same place. The fresh skins weighed all together 206 gm, and the average weight of a skin was 0.43 gm. The physalaemin content was 150 μg per gram fresh skin.

(c) 275 adult specimens captured in part near Tucuman and in part near Cordoba in December, 1963. The total weight of the dried skins was 43 gm, and the average weight of a single skin was 0.16 gm. The physalaemin content was 700 μg per gram dry skin.

Having found that one dried skin contained exactly the same amount of active principle as one fresh skin, methanol extracts of lots a and b were combined to form the *standard extract Physalaemus 1963*, which was used in the present investigation. The concentration of this extract was always expressed in terms of fresh skin.

Extracts of fresh skins were prepared in Argentina by extracting twice with 5 parts (w/v) of methanol the skins removed from the animals immediately after their sacrifice. The skins selected to be dried were carefully spread out and

[1] This work was supported in part by a grant from the Consiglio Nazionale delle Ricerche, Roma.

were dried in the shade. Immediately after their arrival in Italy, by air mail, they were minced with scissors and then immersed in 10–15 parts of 80% methanol. The liquid was decanted after a week, and the skins were extracted for another week with 15 parts of the solvent. The methanol extracts, yellow in color, were combined and filtered. When kept in dark bottles and refrigerated, they may be stored for months and even years without appreciable loss of activity.

The two test systems most frequently used in the bioassay of physalaemin, owing to their high sensitivity and the excellent dose-response relationship, were the dog blood pressure and the isolated rabbit large intestine. The threshold dose of pure natural physalaemin capable of lowering the blood pressure of the anesthetized dog by intravenous injection is of the order of 1 ng per kilogram; the threshold dose capable of stimulating the isolated rabbit large intestine is 0.5–1 ng per milliliter. Other preparations and test systems which were used in the bioassay of physalaemin, especially in parallel assays, were the rabbit blood pressure, the chicken blood pressure, the isolated guinea-pig ileum, and isolated intestinal and/or uterine smooth muscles from rabbits, rats, dogs, and pigeons (5, 6).

The techniques and materials used for the purification and the degradative steps of physalaemin were essentially the same as those previously adopted for similar experiments with eledoisin (3), except that in all cases the peptide fragments obtained upon partial enzymic or chemical cleavage were separated by high voltage electrophoresis on thick Macherey No. 214 paper (from Nagel and Co., Düren) previously exhaustively washed with a 50% ethanol-water mixture. An apparatus from L. Hormuth, Inh. W. E. Vetter (Heidelberg), according to Wieland and Pfleiderer, was used for all the electrophoresis experiments.

Isolation of Physalaemin

Adsorption on alkaline alumina. In a typical experiment, 675 ml of the *Physalaemus* 1963 standard extract, corresponding to 75 gm fresh skin, was evaporated, under reduced pressure and at 45–50°C, to 50–60 ml, and the remaining aqueous liquid was extracted repeatedly with petroleum ether in order to remove fats. The distillation was then continued until the residue was of syrupy consistence. The residue (18 mg per gram fresh tissue) was taken up in a warm water bath by stirring in 100 ml of 95% ethanol. The abundant brownish precipitate found after storage overnight in the refrigerator was discarded, and the liquid was passed through a column of 140 gm (21 × 3.3 cm) of alkaline alumina. Development of the chromatogram was effected by successive addition of 200–300 ml each of 95, 90, 80, 70, 60, 50, 40, and 30% ethanol, and 200 ml of distilled water. Fractions of 100 ml were collected. Bioassay showed that 60–70% of the physalaemin activity appeared in the 60% ethanol eluates, more precisely in eluates 60B and 60C (Fig. 1).

Two minor polypeptides, active on the rat colon and the rat estrous-uterus but with no or negligible activity on the rabbit large intestine, were eluted by 80 and 70% ethanol, respectively. Their pharmacological study is in progress.

Paper chromatography and electrophoresis soon demonstrated that the spot containing the physalaemin activity gave sharp positive reactions not only to ninhydrin and chlorine, but also to the iodoplatinate reagent of sulfur amino acids (7) and the α-nitroso-β-naphthol reagent of tyrosine (8).

The only biogenic amine occurring in minute amounts (2.5 μg per gram fresh tissue) in the *Physalaemus* skin was leptodactyline. It was eluted by 95% ethanol.

Countercurrent distribution. On the whole an amount of material equiactive to roughly 150 gm fresh tissue was obtained from three alumina columns. It had a dry weight residue of approximately 120 mg, i.e., 0.8 mg per gram fresh tissue.

For further purification, aliquots of this material containing the activity of about 50 gm fresh tissue were evaporated to dryness under reduced pressure and then submitted to a double countercurrent distribution of 40 transfers in a hand-operated train with 25 ml of lower and upper phases capacity. The systems used were respectively *n*-butanol–ethanol–acetic acid–water (80:16:16:128) with a pH of 2 in the lower phase for the first, and *n*-butanol–pyridine–water–0.1 *N* ammonia (68:41:150:2) with a pH of 9 in the lower phase, for the second separation. The active material showed distribution coefficients of 1.1 in the first, and 1.3 in the second system and was recovered at the end of the second run with a yield of activity of 70–75%. An amount of this purified product equiactive to 1 gm fresh tissue had a dry weight residue averaging from 80 to 100 μg and contained 65–70 μg of physalaemin as shown by quantitative amino acid analysis.

On high-voltage paper electrophoresis, the active spot migrated toward the cathode at acidic pH, it's relative position being $E_{1.9}$ = 0.43 Glu (7), and showed no mobility at pH 5.8, indicating an almost neutral isoelectric point. On ascending paper chromatograms it had an R_f value of 0.41 in the system *n*-butanol–acetic acid–water (40:10:50), and of 0.38 in the system *sec*-butanol–1.5 *N* ammonia (75:25). A single active spot com-

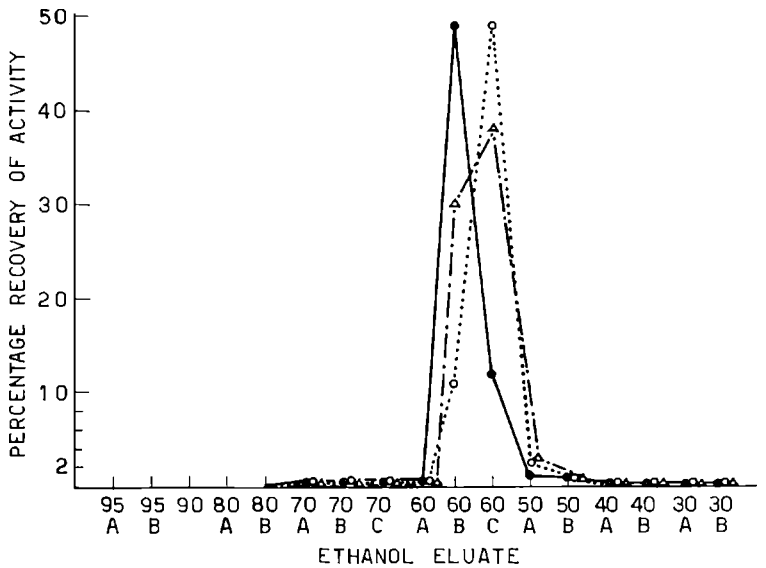


FIG. 1. Elution of physalaemin from three different alkaline alumina columns with descending concentrations of ethanol. In all three experiments the peak of activity, as assessed on the rabbit large intestine, appeared in the 60% ethanol eluates.

pletely free of contaminants was obtained in all the systems tested.

Determination of Amino Acid Composition

Portions of the pure product having the activity of 2–3 gm fresh skin were hydrolyzed with 6 *N* HCl at 105°C in tubes sealed under nitrogen for 18–20 hours, and the reaction mixtures were subjected to bidimensional chromatography run with high-voltage electrophoresis in the first dimension and ascending chromatography in the *n*-butanol–acetic acid–water system in the second dimension. The amino acids found in the hydrolyzate were glutamic and aspartic acids, alanine, proline, lysine, phenylalanine, tyrosine, glycine, leucine, and methionine. Owing to the scarcity of material, only a paper method could be used for quantitative amino acid analysis. However, sufficiently accurate results were obtained by repeating the experiments several times in comparison with standard amounts of pure amino acids subjected to parallel experiments of bidimensional chromatography, elution of the spots and determination of the color intensity. On the other hand, the stepwise enzymic and chemical degradation described in the next section fully confirmed the amino acid composition of the total acid hydrolyzate.

Amino acid estimation was carried out with the method described by Blackburn and Lee (9) for all the amino acid residues except proline, which was estimated after reaction with isatine upon direct comparison with spots produced by different amounts of standard proline. The results showed that nine out of ten residues were present in a 1:1 mole ratio; only the aspartyl residue was present in a 2:1 mole ratio with respect to the other amino acids.

Determination of Amino Acid Sequence

Physalaemin, like eledoisin (2, 3) is resistant to enzymic and chemical attack at both the C and N terminus. Although a number of attempts were made, it was not possible to split the N or C terminal residues with either leucinamino-peptidase or carboxypeptidase digestion; correspondingly no loss of biological activity was observed in the active material exposed to the action of the enzymes for periods of time as long as 15 hours. Also the chemical techniques with fluoro-2,4-dinitrobenzene and phenylisothiocyanate failed in degrading the molecule at the N side.

The amino acid sequence of physalaemin was elucidated by enzymic cleavage with chymotrypsin and trypsin followed by the analysis of the resulting fragments. Indicative results were also obtained with partial acid hydrolysis of the total molecule and of the N-terminal tryptic fragment. The status of the ionic groups was deduced from the migration speed of both the intact molecule and the peptide fragments, subjected to high-voltage electrophoresis in different systems.

Enzymic hydrolyses were carried out with 0.1–0.3 μmole of physalaemin in 0.2 ml of 0.1 *M* ammonium acetate at pH 8.2 containing the enzymes in a weight ratio 1:100 to 1:10. The mixtures were kept in small tubes in a water bath at 37°C for increasing intervals, and were then evaporated to dryness after neutralization with dilute acetic acid. Complete removal of the ammonium salt was obtained by keeping the tubes under a current of nitrogen for 15 minutes at 40°C. The residues were then submitted to electrophoresis. In order to attain the complete cleavage of the susceptible bonds and consequently an optimal separation of the fragments for further study, the mixtures were usually incubated overnight in the presence of the enzymes in a 1:50 ratio.

Complete loss of biological activity resulted upon exposition of physalaemin to the action of both chymotrypsin and trypsin. In this respect the behavior of physalaemin differs from that of eledoisin which does not lose its activity completely when treated with trypsin (3, 10).

Fission with chymotrypsin. Upon chymotryptic digestion the consecutive appearance of the following fragments was observed in the reaction mixtures:

C_1 [Glu, Ala, 2 Asp, Pro, Lys, Phe] Tyr-OH	C_2 [Gly, Leu, Met]
C_3 [Glu, Ala, 2 Asp, Pro]	C_4 [Lys, Phe] Tyr-OH
C_5 H-Lys-Phe-OH	C_6 H-Tyr-OH
C_7 H-Gly-Leu-OH	C_8 H-Met-NH_2

Peptide C_1 was found in the reaction mixtures during the first 2 hours of digestion, and then it disappeared and peptides C_3 and C_4 took its place. Fragments C_5 and C_6 were clearly produced by further degradation of C_4. However, the cleavage of tyrosine from C_4 was very slow, and the presence of the trypeptide was still observed after reaction times of 24 hours. The C terminal position of tyrosine in fragments C_1 and C_4 was deduced from the specificity of the enzyme and from the production of C_5 and C_6 (free tyrosine) upon further degradation of C_4. Therefore the sequence H-Lys-Phe-Tyr-OH of the central tripeptide could be formulated, and the suggested sequence was confirmed by the action of carboxypeptidase on C_1 which led to the splitting in order of time of tyrosine and phenylalanine.

Fragments C_7 and C_8 were split products of C_2, but they were found only in trace amounts even after digestion periods as long as 40 hours. The spot of C_2 was yellow after reaction with ninhydrin, indicating an N-terminal position of glycine (11, 12); furthermore dinitrophenylglycine was obtained upon reaction of C_2 with fluoro-2,4-dinitrobenzene. On the other hand, methioninamide was clearly, although very slowly, liberated from C_2, and therefore it seemed evident that the sequence H-Gly-Leu-Met-NH_2 could be assigned to C_2.

Fragment C_3 showed no migration at acidic pH; it was negative to ninhydrin and positive to chlorine reaction, indicating a structure acylated at the N-terminus.

From the above results the following scheme of action of chymotrypsin may be suggested:

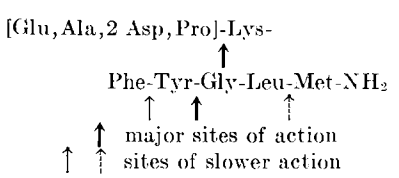
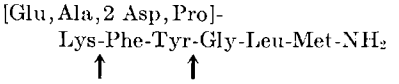


Figure 2 shows the electrophoretic pattern of chymotryptic hydrolysates after 2, 7, and 24 hours of digestion.

Fission with trypsin. Upon digestion with trypsin a result was obtained which could not be explained with the known specificity of the enzyme since in the tryptic hydrolyzates the presence of the following three peptides was observed:

T_1 [Glu, Ala, 2 Asp, Pro]Lys-OH
T_2 [Phe, Tyr]
T_3 [Gly, Leu, Met]

T_3 has the same composition as C_2; therefore the action of trypsin had to be the following



The electrophoretic pattern of the tryptic hydrolyzate is shown in Fig. 3.

Although no systematic experiments on the rate of hydrolysis were made, it could be seen that trypsin cleaved both the -Tyr-Gly- and the -Lys-Phe- bonds with the same speed, and that the action of chymotryptic type at the carboxyl bond of tyrosine was as rapid as that due to the same amount of pure chymotrypsin. The presence of the trypeptide H-Gly-Leu-Met-NH_2 could in fact be observed after 5 minutes of incubation in a mixture containing trypsin in a 1:100 ratio with respect to peptide. After 2 hours the splitting of the C-terminal tripeptide was complete in the same mixture. A small chymotryptic activity of trypsin has already been observed and ascribed to contamination with chymotrypsin or to inherent chymotryptic action (13–15). If the behavior of trypsin with physalaemin was to be attributed to the presence of chymotrypsin, the contamination seemed to be excessive. In order

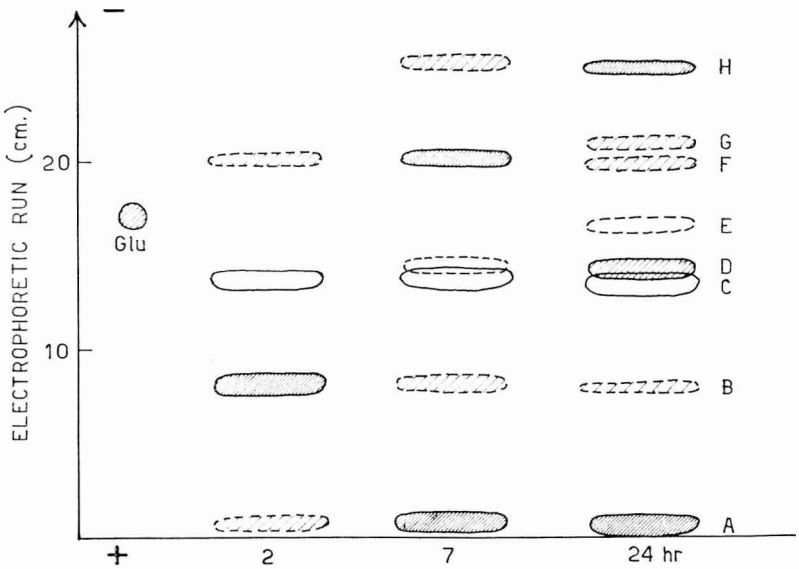


FIG. 2. High-voltage electrophoresis at pH 1.9 of a chymotryptic digest of physalaemin. Voltage gradient, 45 V per centimeter. Electrophoretic pattern after 2,7, and 24 hours of digestion. Band A (origin) stained only with chlorine, Pyr-Ala-Asp(OH)-Pro-Asp(NH_2)-OH; band B ($E_{1.9}$ = 0.47 Glu), Pyr-Ala-Asp(OH)-Pro-Asp(NH_2)-Lys-Phe-Tyr-OH; band C ($E_{1.9}$ = 0.83 Glu), H-Gly-Leu-Met-NH_2; band D ($E_{1.9}$ = 0.85 Glu), H-Tyr-OH; band E ($E_{1.9}$ = 0.95 Glu), H-Gly-Leu-OH; band F ($E_{1.9}$ = 1.2 Glu), H-Lys-Phe-Tyr-OH; band G ($E_{1.9}$ = 1.3 Glu), H-Met-NH_2; band H ($E_{1.9}$ = 1.9 Glu), H-Lys-Phe-OH.

to check the results three different batches of the enzyme thrice crystallized were tested, two of them from Fluka A. G., Buchs, Switzerland and one from Princeton Laboratories, Princeton, New Jersey. All of them behaved in exactly the same way with physalaemin and with the synthetic peptide, H-Phe-Tyr-Gly-Leu-Met-NH_2, but they showed no chymotryptic action on other substrates specific for chymotrypsin (e.g., bradykinin).

Finally it was seen that both the specific and the aspecific splitting by trypsin of the -Lys-Phe- and the -Tyr-Gly- bonds could be inhibited by the specific soybean trypsin inhibitor (100 μg physalaemin in 0.2 ml of 0.1 *M* ammonium acetate at pH 8.2 + 50 μg trypsin + 100 μg soybean inhibitor, 4 hours at 37°C).

Incubation of T_1 with carboxypeptidase-B caused the rapid release of lysine and the production of a fragment on which carboxypeptidase-B and carboxypeptidase-A had no further action. It was clearly the same fragment C_3 produced by chymotrypsin which was unreactive to ninhydrin and showed the electrophoretic mobility of a negatively charged peptide.

With the above results the sequence -Lys-Phe-

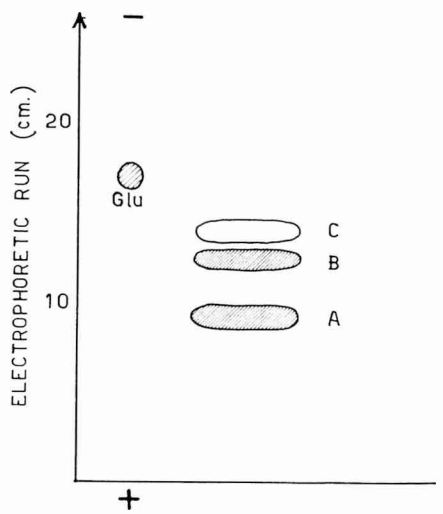


FIG. 3. High-voltage electrophoresis at pH 1.9 of a tryptic digest of physalaemin. Voltage gradient, 45 V per centimeter. Band A ($E_{1.9}$ = 0.56 Glu), Pyr-Ala-Asp(OH)-Pro-Asp(NH_2)-Lys-OH; band B ($E_{1.9}$ = 0.74 Glu); H-Phe-Tyr-OH; band C ($E_{1.9}$ = 0.83 Glu), H-Gly-Leu-Met-NH_2.

Tyr-Gly-Leu-Met-NH_2 could be assigned to the C-terminal hexapeptide. The sequence of the N-terminal pentapeptide [Glu,Ala,2 Asp,Pro] was elucidated on the basis of the data obtained with partial acid hydrolysis experiments.

Partial acid hydrolysis. The hydrolyses were carried out with 0.2 μmole of physalaemin or with an amount of fragment T_1 produced by tryptic hydrolysis of 0.4 μmole of physalaemin. The samples were dissolved in 0.3 ml of 0.5 *M* acetic acid and were allowed to stand in sealed tubes at 100°C for 15 and 40 hours. It was soon evident that physalaemin was split in essentially the following way with release of free aspartic acid

[Glu,Ala] H-Asp(OH)$_2$
[Pro,Asp]-Lys-Phe-Tyr-Gly-Leu-Met-NH_2

while the splitting products of T_1 were consecutively

Ac_1 [Glu, Ala] → Pyr-OH, H-Ala-OH
H-Asp-(OH)$_2$
Ac_2 [Pro, Asp] Lys-OH → Ac_3 [Pro, Asp] H-Lys-OH

Ac_1 was negative to ninhydrin and migrated toward the anode when submitted to electrophoresis at pH 5.8. Upon further action of dilute acid it gave alanine and a spot negative to ninhydrin which was readily identified as pyroglutamic acid.

Ac_2 showed the electrophoretic mobility of a positively charged peptide in acid and neutral medium, while the mobility of Ac_3 indicated that it was positively charged at pH 1.9 and neutral at pH 5.8. Therefore it could be concluded that they contained an asparaginyl residue. Finally both Ac_2 and Ac_3 gave positive reactions with isatine, indicating an N-terminal position of proline.

The above results were unequivocally conclusive for the assignment of the N-terminal structure, and consequently the complete sequence of physalaemin was formulated as follows:

Pyr-Ala-Asp(OH)-Pro-Asp(NH_2)-Lys-Phe-Tyr-Gly-Leu-Met-NH_2

Identity of Pure Natural Physalaemin with the Active Peptide Present in Crude Skin Extracts and with Synthetic Physalaemin

It is known that an N-terminal glutamine can be converted into a pyroglutamic residue during the isolation procedures (16–18). Thus the problem arose, exactly as in the case of eledoisin, of whether the endecapeptide obtained following the purification procedures described herein possessed the same N-terminal structure of the peptide present in the crude methanol extracts of the skin. We attempted to solve this problem by paper electrophoretic experiments and by parallel biological assays of different physalaemin preparations in numerous test systems.

Amounts of crude methanol extracts corresponding to 1 mg fresh skin were subjected to high-voltage paper electrophoresis in parallel with 1 μg of pure natural physalaemin and 1 μg of pure synthetic physalaemin. It was found that the relative position on pherograms of the active spots was always the same ($E_{1.9}$ = 0.43 Glu). Moreover, electrophoresis of mixtures of crude methanol extract and pure physalaemin always gave a unique active spot.

The following four physalaemin preparations were subjected to parallel bioassay: (a) the crude standard *Physalaemus* extract 1963; (b) a 60% ethanol eluate from an alumina column; (c) pure natural physalaemin; and (d) synthetic physalaemin purified by two successive countercurrent distributions.

The three first preparations were suitably diluted so that they possessed exactly the same hypotensive action in the dog. Synthetic physalaemin was always used at the same concentration as natural physalaemin. These preparations were then assayed in parallel on rabbit blood pressure, chicken blood pressure, rabbit large intestine, guinea-pig ileum, and pigeon duodenum. The activity of the *Physalaemus* standard extract was always considered equal to 100, and the activity of the other preparations was expressed in per cent.

Table I clearly shows that doses of the three preparations of natural physalaemin which are equiactive on the dog blood pressure are also equiactive in all other test systems, i.e., that the pure natural polypeptide is indistinguishable, by bioassay also, from the polypeptide present in crude skin extracts. It further shows that synthetic physalaemin is identical with natural physalaemin.

In connection with the problem discussed in this section, it seems pertinent briefly to describe some recent experiments on eledoisin.

Crude methanol extracts of posterior salivary glands of *Eledone* (10) corresponding to 1–2 mg fresh tissue were subjected to high-voltage paper electrophoresis in parallel with pure natural eledoisin, pure synthetic eledoisin, and synthetic glutamine eledoisin, Glu(NH_2)-Pro-Ser-Lys-Asp(OH)-Ala-Phe-Ile-Gly-Leu-Met-NH_2. Whereas the position of the active polypeptide spots given by the three first preparations was exactly the same ($E_{1.9}$ = 0.43 Glu), glutamine

TABLE I

RESULT OF PARALLEL BIOASSAY OF FOUR PHYSALAEMIN PREPARATIONS ON SEVEN TEST OBJECTS[a]

Test object	Relative activity (%)			
	Physalaemus 1963 standard extract	60% ethanol eluate	Pure natural physalaemin	Synthetic physalaemin
Dog blood pressure	100	100	100	100
Rabbit blood pressure	100	105–115	100–110	95–105
Chicken blood pressure	100	90	110	100
Rabbit large intestine	100	105	100–105	95–105
Guinea-pig ileum	100	105	95–100	95–105
Dog large intestine	100	95	95	—
Pigeon duodenum	100	95	—	—

[a] The activity of the *Physalaemus* 1963 standard extract was always considered equal to 100; that of the other three physalaemin preparations was expressed in per cent.

eledoisin, as expected, migrated toward the cathode faster than eledoisin ($E_{1.9}$ = 0.76 Glu). On the other hand, solutions of glutamine eledoisin in ethanol or methanol have been stored for months without any appreciable transformation of the N-terminal glutamine into a pyroglutamic residue. The conclusion from these two sets of experiments is that eledoisin is the very polypeptide present in the fresh salivary tissue of *Eledone*.

Although we have as yet not had the opportunity of examining glutamine physalaemin, it seems that the above conclusions for eledoisin may be analogically extended to physalaemin.

DISCUSSION

The most interesting fact which has emerged from the present study is that of the strict chemical resemblance existing between physalaemin (I) and eledoisin (II)

(I) Pyr-Ala-Asp(OH)-Pro-Asp(NH_2)
-Lys-Phe-Tyr-Gly-Leu-Met-NH_2

(II) Pyr-Pro-Ser-Lys-Asp(OH)
-Ala-Phe-Ile-Gly-Leu-Met-NH_2

This resemblance satisfactorily explains the striking similarity in the biological effects of the two polypeptides, which has allowed us to ascribe to physalaemin the attribute "eledoisin-like" long before the elucidation of its chemical structure was accomplished (1).

The two above formulations show that the N-terminal amino acid residue is the same for physalaemin and eledoisin and, what is still more important, at least from the point of view of biological activity, the C-terminal tripeptide is exactly the same also. Yet eledoisin is found in the salivary glands of an invertebrate, and physalaemin in the skin of a vertebrate.

Something similar has been observed in the case of the bradykinins. The amphibian skin contains a number of bradykinin-like polypeptides, but one of them, the bradykinin of *Rana temporaria* skin, is identical to the bradykinin originating in human blood by the action of proteolytic enzymes on plasma bradykininogen (19).

The major difficulty in approaching the study of the structure of physalaemin was the scarcity of material, since about 15–18 mg of pure peptide were available at the end of the purification procedures. The high-voltage electrophoresis technique was of valuable help in overcoming the difficulties, as it permitted a good separation of the degradation products of very small samples of active peptide.

As already stated, the correctness of the proposed structure of physalaemin has been recently checked by comparing the biological activity, the physicochemical properties and the behavior toward chemical and biochemical degradative agents of the natural product with the properties of a synthetic preparation of the endecapeptide (20).

It has been pointed out that the behavior of physalaemin toward trypsin was in part quite unexpected. In an attempt to interpret the observed divergencies from the generally accepted schemes, we are now

carrying out experiments on trypsin fission of synthetic analogues of physalaemin. The anomalous cleavage by trypsin of two bonds, instead of one, in the physalaemin molecule explains the complete loss of activity of this polypeptide produced by trypsin. On the other hand, the normal cleavage of only one bond, between lysine and aspartic acid, in the eledoisin molecule, with consequent release of a fairly large heptapeptide fragment, explains why eledoisin retains 10–15% of its biological activity after trypsin digestion.

Some features of chymotryptic hydrolysis are also noteworthy. When the complete sequence of physalaemin was known, it became evident that chymotrypsin caused the fairly unusual quantitative splitting at the carboxyl bond of asparagine and a partial splitting of adjacent aromatic residues. Other workers have already observed similar behavior (21–24). It may be suggested that the slow cleavage of the phenylalanyl bond in physalaemin is due to inhibition by the adjacent free carboxyl produced by the faster rupture of the tyrosine bond.

Although eledoisin and physalaemin possess the same C-terminal tripeptide sequence, methioninamide is released rapidly and quantitatively from eledoisin, while it is very slowly and only in trace amounts liberated from physalaemin. This is probably due to the fact that, whereas the tetrapeptide, -Ile-Gly-Leu-Met-NH_2, which is first released from eledoisin, is susceptible to further easy hydrolysis by chymotrypsin, the tripeptide -Gly-Leu-Met-NH_2 produced by the major attack of the enzyme on the physalaemin molecule does not seem to constitute a good substrate for further hydrolysis. Experiments of digestion with chymotrypsin of the synthetic C-terminal tripeptide have confirmed this interpretation.

REFERENCES

1. ERSPAMER, V., BERTACCINI, G., AND CEI, J. M., *Experientia* **18,** 562 (1962).
2. ERSPAMER, V., AND ANASTASI, A., *Experientia* **18,** 58 (1962).
3. ANASTASI, A., AND ERSPAMER, V., *Arch. Biochem. Biophys.* **101,** 56 (1963).
4. ERSPAMER, V., ANASTASI, A., BERTACCINI, G., AND CEI, J. M., *Experientia* **20,** 489 (1964).
5. ERSPAMER, V., AND FALCONIERI ERSPAMER, G., *Brit. J. Pharmacol.* **19,** 337 (1962).
6. ERSPAMER, V., AND GLÄSSER, A., *Brit. J. Pharmacol.* **20,** 516 (1963).
7. GUTMANN, S., AND BOISSONNAS, R. A., *Helv. Chim. Acta* **41,** 1852 (1958).
8. ACHERS, R., AND CROCKER, C., *Biochim. Biophys. Acta* **9,** 704 (1952).
9. BLACKBURN, S., AND LEE, C. R., *Biochem. J.* **87,** 1P (1963).
10. ANASTASI, A., AND ERSPAMER, V., *Brit. J. Pharmacol.* **19,** 326 (1962).
11. CONSDEN, R., GORDON, A. H., AND MARTIN, A. J. P., *Biochem. J.* **41,** 590 (1947).
12. KNIGHT, C. A., *J. Biol. Chem.* **190,** 753 (1951).
13. BROMER, W. W., STAUB, A., SINN, L. E., AND BEHRENS, O. K., *J. Am. Chem. Soc.* **79,** 2801 (1957).
14. MCFADDEN, R., LASKOWSKI, M., *130th Meeting Am. Chem. Soc.* Abstr. 71C (1956).
15. KIMMEL, J. R., KATO, G. K., PAIVA, A. C. M., AND SMITH, E. L., *J. Biol. Chem.* **237,** 2525 (1962).
16. GISH, D. T., *Biochim. Biophys. Acta* **35,** 557 (1959).
17. DEKKER, C. A., STONE, D., AND FRUTON, J. S., *J. Biol. Chem.* **181,** 719 (1949).
18. RUDINGER, J., AND PRAVDA, Z., *Collection Czech. Chem. Commun.* **23,** 1947 (1958).
19. ANASTASI, A., ERSPAMER, V., AND BERTACCINI, G., *Comp. Biochem. Physiol.* (In press).
20. BERNARDI, L., BOSISIO, G., GOFFREDO, O., AND DE CASTIGLIONE, R., *Experientia* **20,** 490 (1964).
21. KONIGSBERG, W., HILL, R. J., *J. Biol. Chem.* **237,** 3156 (1962).
22. HIRS, C. H. W., MOORE, S., AND STEIN, W. H., *J. Biol. Chem.* **235,** 633 (1960).
23. MATSUHARA, H., AND SMITH, E. L., *J. Biol. Chem.* **237,** PC 3575 (1962).
24. GINSBURG, A., AND SCHACHMAN, H. K., *J. Biol. Chem.* **235,** 108 (1960).